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Long-Term Culture of Lobster Central Ganglia: Expression of Foreign Genes in Identified Neurons

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Abstract. The ventral nerve cords of lobsters (*Homarus americanus*) can be cultured *in vitro* for at least 7 weeks. Over this period, neurons maintain their normal electrophysiological features and continue, among other measures of neuronal health, to synthesize RNA and proteins. One application of this culture system is demonstrated: the manipulation of gene expression in identified neurons. After intracellular injection of complementary RNA (cRNA) encoding green fluorescent protein (GFP), the amount of protein product measured by fluorescent confocal microscopy increases for 4 days and then decreases to background by day 10. Thus, translation of the injected message must have increased for 4 days before declining. Moreover, after injection of cRNA encoding β-galactosidase, the levels of enzyme activity were measured using a fluorogenic substrate, revealing a peak of β-galactosidase activity at 6 to 9 days; this activity was still detectable for at least 10 days after injection.

Therefore, either GFP or β-galactosidase can be used as an injectable marker, allowing *in vivo* quantitation of expression in individual cells over time. We measured long-lasting expression of these proteins after a single injection, suggesting that it may be possible to manipulate the levels of expression of any gene of interest.

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* This paper is dedicated to the memory of a good colleague and friend, Dr. Richard P. Bunge. Dick died on September 10, 1996, of esophageal cancer at the age of sixty-four. He was in the midst of an important project, that of rebuilding the Miami Project to Cure Paralysis, and in 1989 became the scientific director of that project. One of us (EAK) had the good fortune to work with Dick from 1968 to 1969 during his sabbatical visit to our laboratory in Boston. The organ culture system was developed at that time, and although these earlier experiments never were published, they are an important part of our present and future research activities. It is typical of Dick and his studies that they often were far ahead of their time. We are honored to include him as an author on this paper.

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Introduction

The ventral nerve cord preparation has been a useful tool for exploring the physiology and pharmacology of central neurons in the lobster (see Otsuka *et al.*, 1967; Livingstone *et al.*, 1980; Ma and Weiger, 1993). The entire nerve cord, with the nerve roots sectioned, can be dissected from lobsters and maintained in oxygenated saline for up to 36 hours. After the ganglia have been desheathed, the large cell bodies of central neurons can be identified both by their positions and by their physiological properties as revealed in electrophysiological recordings.

In this study, we report an extension of this technique that allows experiments of at least 7 weeks duration to be carried out. Longer term experiments that become possible under these conditions include an exploration of the effects of manipulation of levels of gene expression. Since activation of a gene can take several days to affect cells, experiments of this type were not practical with the short-term preparations that are commonly used.

Genes from practically any source can be injected into *Xenopus* oocytes and the resulting heterologous expression analyzed (for review, see Dawid and Sargent, 1988). Use of this technique allows, for example, the effects of point mutations or deletions in genes to be examined. Although expression can be characterized in heterologous systems, the function of cloned genes might be more informatively explored if they could be expressed in cells of their native organism. Our studies show that this important option is

now available for investigations of the roles of neuronal genes in the central neurons of the lobster. Moreover, we can use this technique to express proteins or peptides in neurons and investigate both the physiological responses of the neuron and the effectiveness of the introduced substance.

Injection of DNA or RNA into identified neurons can be a direct and effective means of altering gene expression in identified cells. Several investigators have used microinjection techniques to express genes in invertebrate neural systems; in particular, the effects of various proteins on synaptic transmission and electrical excitability of neurons have been determined. For example, Kaang and coworkers (1992) found that microinjection of DNA encoding an A-type Shaker potassium channel into *Aplysia* neurons shortened action potentials and thereby had a profound effect on transmitter release. Expression of a noninactivating potassium channel in another type of *Aplysia* neuron abolished spontaneous bursting activity (Zhao *et al.*, 1994). RNA encoding the leech homeobox protein Lox1, when injected into certain types of leech motoneurons, introduced active spike propagation to proximal neurites (Aisemberg *et al.*, 1997). In another system, Dearborn and coworkers (1998) injected rat synapsin 1A1 into crayfish neurons and found an increase in transmitter release.

In this communication, we present an organ culture system that extends the time frame of nerve cord experimentation, allowing for such long-term experiments as the expression of foreign RNAs in lobster central neurons. We have induced the expression of green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (Prasher *et al.*, 1992; Chalfie *et al.*, 1994) and the enzyme β-galactosidase from the bacterium *Escherichia coli* (MacGregor *et al.*, 1987). Levels of both proteins were measured repeatedly in individual living cells over several days. Neither of these proteins interfered with the viability of the cells. These reagents can be injected either as continuous expression monitors or as fusion proteins linked with other proteins of interest (Lalumiere and Richardson, 1995; Gerdes and Kaether, 1996). This study confirms the effectiveness of the gene delivery system and our long-term organ culture system, a combination that promotes connections between the disciplines of molecular biology and physiology in the study of the lobster nervous system.

Materials and Methods

Dissection and neuron identification

Nerve cords were removed from ice-anesthetized adult lobsters and pinned out in lobster saline as previously described (Otsuka *et al.*, 1967; Harris-Warrick and Kravitz, 1984; Ma *et al.*, 1992). After desheathing the abdominal ganglia and removing the glial layers, we targeted two cell types for injections. These were identified according to criteria defined by Otsuka *et al.* (1967) and included large glutamate-containing motoneurons (M6/M7), with their axons projecting through ipsilateral 3rd roots, and GABA-containing neurons (I2), displaying prominent excitatory synaptic input and projecting through contralateral 3rd roots to the periphery.

Lobster organ culture system

After experimental manipulation, preparations were rinsed several times in sterile saline containing penicillin (50 μg/ml), streptomycin (50 μg/ml) and neomycin (100 μg/ml) (GIBCO/BRL). Nerve cords were rinsed twice in a modified Leibovitz's L-15 medium, pinned out in ethanol- and UV-sterilized Sylgard-coated (Dow Corning) petri dishes (60 mm diameter), and then incubated in the modified medium. The culture medium contained Leibovitz's L-15 medium with L-glutamine at 300 mg/l (GIBCO/BRL) and with the following additions made to bring the salt concentration to levels suitable for incubation of lobster tissues: NaCl was adjusted to 462 m*M*; KCl to 16 m*M*; $CaCl_2$ to 26 m*M*; $MgCl_2$ to 8 m*M*; and glucose to 11 m*M*; HEPES buffer (pH 7.4) at 10 m*M* (all salts from Sigma); fetal bovine serum (GIBCO/BRL) at a final concentration of 10%. Antibiotics and antifungal agents were added at the same concentrations specified above. Nerve cords were cultured at 16°C in an air incubator (Isotemp, Fisher) and the culture medium was changed twice weekly.

Lobster ganglion labeling and autoradiography

Labeling and autoradiography were carried out according to the method of Hendelman and Bunge (1969). Lobster ganglia were incubated in medium containing 10 μM ^{3}H-uridine (RNA labeling) or 10 μM ^{3}H-leucine (protein labeling) at high specific activity (New England Nuclear). Tissues were fixed in 2% osmium tetroxide buffered with veronal acetate (pH 7.4) with added $CaCl_2$ (0.05%) (all chemicals from Sigma). After several washes, ganglia were dehydrated in a graded ethanol series and embedded in a mixture of 10 parts Epon 812 (Structure Probe, Inc. (SPI)); 10 parts Araldite 6005 (SPI); 24 parts DDSA (dodecenyl succinic anhydride, SPI); and 2% DMP-30 (2,4,6-tris(dimethylaminomethyl)phenol, Sigma). Sections of 1–1.5 μm were cut and autoradiographed at 4°C in Ilford L-4 emulsion (Ferranti-Dege). Slides were developed with D-19 (Kodak) and mounted in glycerin. Two experimental sets were examined in detail using this method. In one, pairs of ganglia were incubated in organ culture for 2 days or for 49 days, before incubation for 4 h in ^{3}H-leucine or ^{3}H-uridine, followed by 20 h of washout in nonradioactive medium, fixation and processing all as described above. In the second experimental set, ganglia were cultured for 30 and 45 days *in vitro* before labeling, fixation, and autoradiography.

GABA measurements

GABA in single cells was measured by the original assay method of Jakoby and Scott (1959). This method uses the enzymes GABA-glutamic transaminase and succinic semialdehyde dehydrogenase to generate spectrophotometrically detectable triphosphopyridine nucleotide (reduced) in direct proportion to the amount of GABA in a sample. Single-cell-body isolation, extraction, and enzyme measurements were carried out as described in Otsuka *et al.* (1967).

RNA reagents

Capped GFP cRNA was transcribed from Xho1-linearized pSEM/GFP (generously provided by Richard Dearborn, see Dearborn *et al.*, 1998) (enzyme from New England Biolabs) using the CapScribe SP6 kit (Boehringer Mannheim) and following the manufacturer's instructions. β-galactosidase-encoding cRNA was transcribed from pCMV-SPORT-β-gal (GIBCO/BRL, linearized with XmnI, New England Biolabs) by the same method. RNA quality and quantity were monitored by gel electrophoresis and UV spectrophotometry. The solutions used for injection contained cRNA at 0.5 to 1 μg/μl and included 0.1 U/μl of RNase inhibitor (RNasin, Promega).

RNA was introduced into the cytoplasm of lobster neurons by pressure injection through an intracellular recording electrode (pressure at compressor: 5–20 psi). Glass capillaries (Drummond Scientific Company) were baked overnight at 300°C to facilitate filling and to remove possible sources of RNase activity; microelectrodes were pulled (Narishige PE-2) and the tips were broken to less than 4 μm by tapping them against a fine glass rod under a compound microscope at 200× magnification. Electrodes were filled by first dipping their tips into 2 μl of the electrode solution (see below) on a small piece of Parafilm (American National Can). The remaining solution was picked up using a pipette tip and injected into the back of the electrode, where it quickly ran to the tip by capillarity. Electrodes were mounted in an electrode holder (MEH2RW, World Precision Instruments (WPI)) fitted with a silver wire long enough to make electrical contact with the solution in the tip of the electrode. The electrode wires were treated with RNaseZap! solution (Ambion), with care taken in loading the RNA solution to avoid RNase contamination. Potassium acetate was added to the RNA samples to a final concentration of 0.5 *M*, along with 0.05% phenol red (Sigma) for visualization. Control injections revealed that phenol red did not interfere with the fluorescence detection or the viability of the cells, and appeared to diffuse out of injected cells within a few hours. Cells were injected slowly with RNA solution until the somata appeared slightly red.

DNA reagents

Plasmid DNA was injected in the same way as was the RNA. Purified DNAs including the following constructs were injected at concentrations ranging from 0.1 to 5 μg/μl: (i) *Drosophila melanogaster* neuron-specific *elav* promoter (Yao and White, 1994) driving a β-galactosidase reporter gene (gift from Dr. Thomas Schwarz); (ii) human CMV immediate early enhancer/promoter (Thomsen *et al.*, 1984) driving a fusion of the human β-2 adrenergic receptor and the *Aequoria victoria* GFP (gift from Dr. Timothy McClintock); and (iii) *Drosophila melanogaster* heat-shock inducible promoter from *hsp70* (Pelham, 1982) driving GFP.

Physiological recordings

Intracellular recordings from neuronal somata were performed with glass microelectrodes filled with 1.0 *M* potassium acetate (12–25 MΩ resistance). For injections, this solution was replaced by a mixture of 0.5 *M* potassium acetate and the molecular constructs to be injected (3–5 MΩ resistance). Electrical signals were amplified with an Axoprobe 1A amplifier (Axon Instruments). The cells were physiologically identified by their synaptic input and antidromic stimulation of their axons in the 3rd roots following criteria defined by Otsuka and coworkers (1967). Nerve roots were stimulated by placing their cut ends into closely fitting suction electrodes. Electrical stimuli were generated and delivered by a Master-8 stimulus generator (A.M.P.I.) with built-in isolation units.

Detection of cRNA expression

Injected neurons were periodically monitored, using confocal fluorescence microscopy, for GFP expression or for β-galactosidase activity. Nerve cords were transferred from culture vessels to sterile, medium-filled, deep-well microscope slides that had a thin coat of Sylgard on their lower surfaces. The cords were pinned out, coverslipped, and observed with a scanning laser confocal microscope equipped with FITC filters (MRC 600, Bio-Rad). For β-galactosidase activity determinations, fluorescein di-(β-D-galactopyranoside)(Sigma) dissolved in dimethyl sulfoxide to make a final concentration of 0.67 mg/ml was added to the medium on ice after the preparation had been transferred to the slide.

For GFP measurements, 6.5-μm optical sections were taken through the cell body region of the injected cell and images recorded using the confocal microscope's photomultiplier tubes at various times after injection. For β-galactosidase measurements, a single section was scanned as quickly as possible after transferring the slide to the microscope, and the increase in fluorescence with time was measured over the next 35–40 min in periodic scans. Fluores-

cence was quantified by measuring average pixel intensity for comparable single sections using NIH Image software (Shareware by Wayne Rasband, National Institutes of Health). Following each imaging session, preparations were rinsed again in sterile saline and lobster L-15 and repinned in the culture dish; the culture was continued at 16°C.

Results

Survival of central neurons in long-term organ culture

The ventral nerve cord was removed from an adult lobster and maintained in a modified Leibovitz L-15 culture medium for at least 7 weeks. Over this period, identified neurons maintained features typical of their condition in the short-term preparation routinely used for physiological experiments (usually from 12 to 36 h).

Physiology of neurons. The positions of the somata of many neurons in the lobster ventral nerve cord have been mapped (Otsuka *et al.*, 1967; Beltz and Kravitz, 1983; Schwarz *et al.*, 1984; Schneider *et al.*, 1993). Cell bodies can be reliably and readily identified from preparation to preparation by their relative sizes, their position in a ganglion, their endogenous and nerve-evoked synaptic input, their spontaneous activity, and by backfiring their axons. To illustrate, I2, a large GABA-containing inhibitory motor neuron innervating the fast flexor muscles, was studied in a preparation maintained for 49 days in culture (Fig. 1). As shown in the schematic, an intracellular recording electrode was placed in the putative I2 cell in this ganglion, and stimulating electrodes were placed on the anterior connective (point 1) and contralateral 3rd root of the ganglion (point 2). Stimulation of the contralateral 3rd root led to the appearance in the cell body of small action potentials (action potentials do not invade these somata) that retained a constant amplitude with increasing intensities of stimulation. No other large cell bodies showing this property were seen in that location, hence the identification of the cell as I2 is highly likely and further supported by the demonstration that these neurons contain GABA. In contrast, with increasing levels of stimulation of the anterior connective (point 1, Fig. 1, left), excitatory postsynaptic potentials measured in I2 increased in amplitude, finally generating action potentials in these cells. This suggests that a progressive recruitment of presynaptic inputs to I2 is occurring, and that these inputs, too, have survived the long-term organ culture. When the connective between electrode 1 and the ganglion was crushed, excitation of I2 *via* this route was abolished (Fig. 1, top right), thereby assuring that the responses seen were not due to current spread from the stimulating electrode. Resting membrane potentials in I2 and other cells in this and in other experiments were within normal ranges (−50 to −65 mV, data not shown). GABA was detected in I2 cell somata dissected from organ-cultured ganglia at concentrations normally found in I2 cell

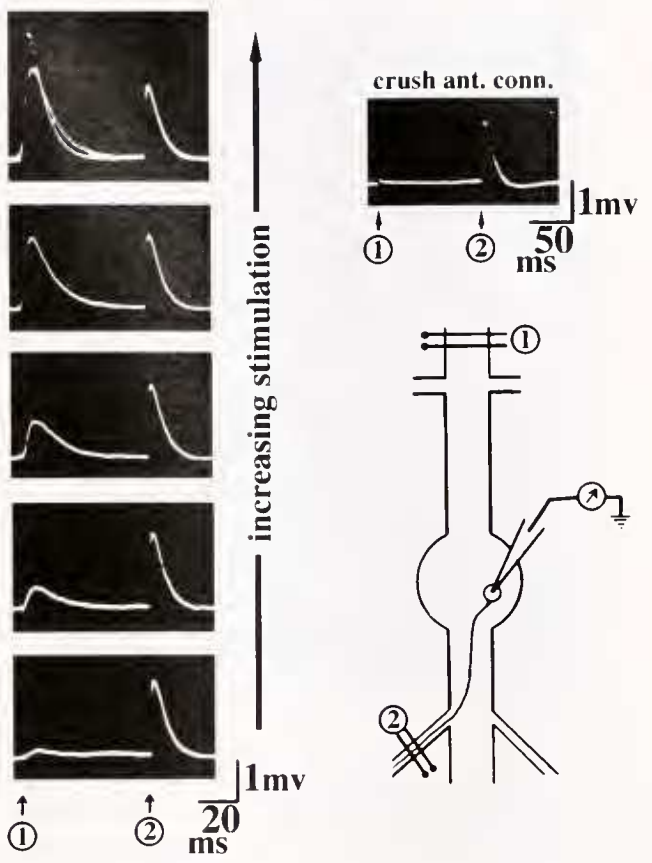

Figure 1. Normal activity in a lobster ganglion maintained in organ culture for 49 days. While recording intracellularly from an abdominal GABA-containing cell, I2, the preparation was stimulated from the anterior connective (point 1). Increasing stimulation intensity (left panel, bottom to top) elicited an increasing excitatory response in I2 (at point 1) that finally triggered an action potential. After the anterior connective was crushed between the stimulating electrode and the ganglion, the EPSPs in I2 disappeared (upper right). When the 3rd root was stimulated directly (point 2 at right), an antidromic action potential was triggered in I2 and recorded in the cell body. As expected, the action potential showed no size change with increasing stimulus intensity.

bodies from freshly dissected preparations [fresh preparations—$0.79 \pm 1.75 \times 10^{-10}$ moles/cell body (mean ± SD, $n = 14$) (Otsuka *et al.*, 1967); 6-week cultures—$1.1 \pm 7.11 \times 10^{-10}$ moles/cell body (mean ± SD, $n = 4$)].

Evidence of RNA synthesis by neurons. RNA synthesis in cultured nerve cords was measured by radiolabeled precursor incorporation and autoradiography. In Figure 2, one example is presented. Lobster ganglia maintained in organ culture for 2 and 49 days were incubated in medium containing ^{3}H-uridine for 4 h, followed by a washout with unlabeled medium for 20 h. Ganglia were fixed, embedded, and sectioned, and autoradiography was performed. Silver grains are readily seen over the nuclei of neurons and glial cells (Fig. 2). These represent the location of newly synthesized RNA and demonstrate that neurons in long-term culture still actively transcribe RNA. Qualitatively, we saw

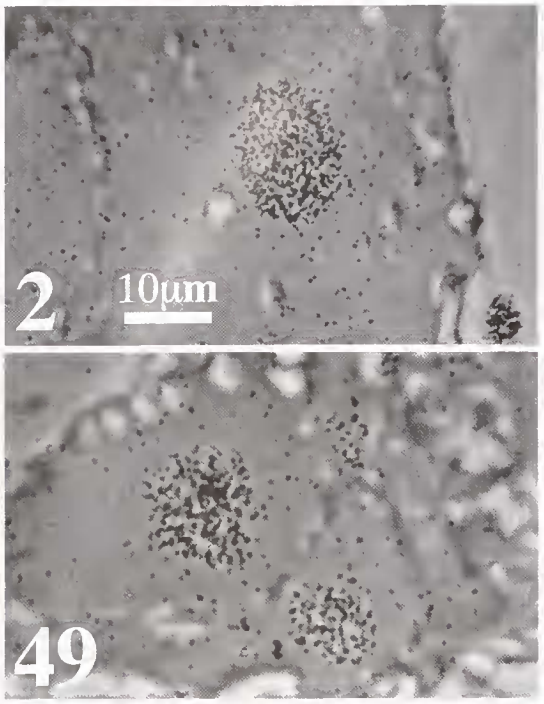

Figure 2. Autoradiography of newly synthesized RNA in lobster ganglia maintained for 2 and 49 days *in vitro*. Ganglia were treated with ^{3}H-uridine for 4 h, followed by a chase with unlabeled medium for 20 h. Following fixation, samples were embedded and sectioned, and autoradiography was performed. The resulting silver grains clustered primarily at the nuclei of cells cultured for 2 and 49 days indicate the presence of nascent RNA.

little difference in grain density between the 2-day and 49-day organ cultures.

Similar labeling experiments were performed after other times of incubation with both ^{3}H-uridine and ^{3}H-leucine. In the latter experiments, a cytoplasmic rather than nuclear distribution of silver grains was noted after autoradiography, indicating the synthesis of protein in the cultured ganglia (data not shown).

Cytology of ganglia. Some indication of overall health can be seen in the cytology of cultured ganglia. Cells of ganglia cultured for the longer time period appeared similar to cells of recently dissected ganglia, although the former usually had larger spaces between cells (not shown). Rarely, opaque or darkly colored cells were observed in cultured lobster ganglia, such as those shown in Figure 3 (bottom panel). These cells typically had low or no resting potentials and were likely to be dead or dying. Another feature of unhealthy cells is their slight autofluorescence (Fig. 3, top). The bright cell in Figure 3, top panel, is a GFP-expressing cell. As seen in bright-field illumination (Fig. 3, bottom panel), the GFP-expressing cell is transparent, whereas the two weakly fluorescent cells are opaque. The death of these two cells resulted from experimental DNA injection, described below. The numbers of spontaneously unhealthy cells was generally low throughout the culture period: most cells and their processes appeared morphologically undistinguishable from their counterparts in freshly dissected ganglia.

Expression of introduced RNAs in organ-cultured neurons

A single injection of cRNA for either GFP or β-galactosidase into central neurons in cultured ganglia led to the synthesis of these proteins. Their presence was detected with fluorescence and confocal microscopy, either directly in the case of GFP, or indirectly for β-galactosidase, by adding a fluorogenic substrate for the enzyme. Measurements were made by periodically locating the same injected cell in organ-cultured cords.

Induction of GFP expression. An identified cell, usually I2, M6, or M7, was pressure-injected with the cRNA for GFP and maintained in organ culture for periods of up to 10 days. At various time points the injected ganglia were imaged using a confocal fluorescence microscope to detect and measure GFP expression. GFP fluorescence in this cell was detected after one day, increased until day 4, then decreased to background

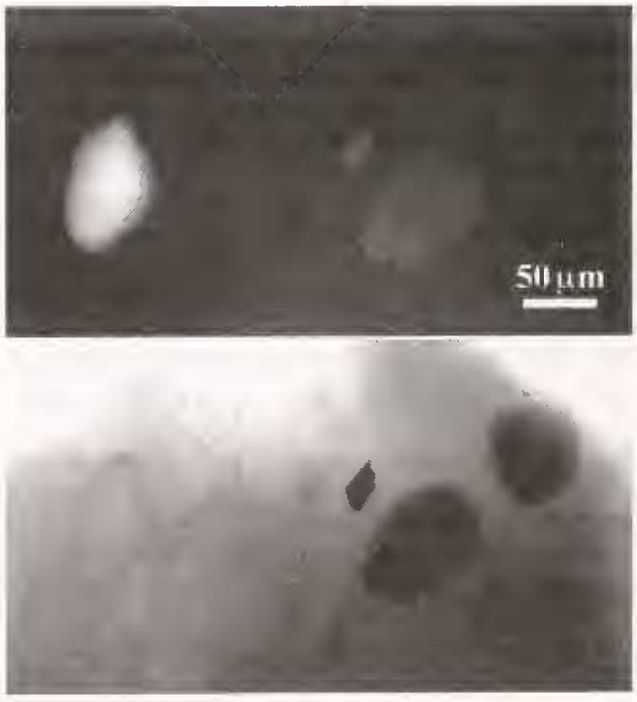

Figure 3. Comparison of a GFP-expressing neuron with two cells injected with high concentrations of plasmid DNA. The highly fluorescent cell seen with epifluorescent illumination (top panel) was injected with cRNA encoding green fluorescent protein (GFP). After 3 days of incubation, this cell appeared clear under bright-field illumination (bottom). In contrast, two cells injected with plasmid DNA fluoresced only weakly (top) and were opaque under bright-field illumination (bottom). These cells had poor (or no) resting membrane potentials and resembled untreated cells that occasionally died during prolonged organ culture.

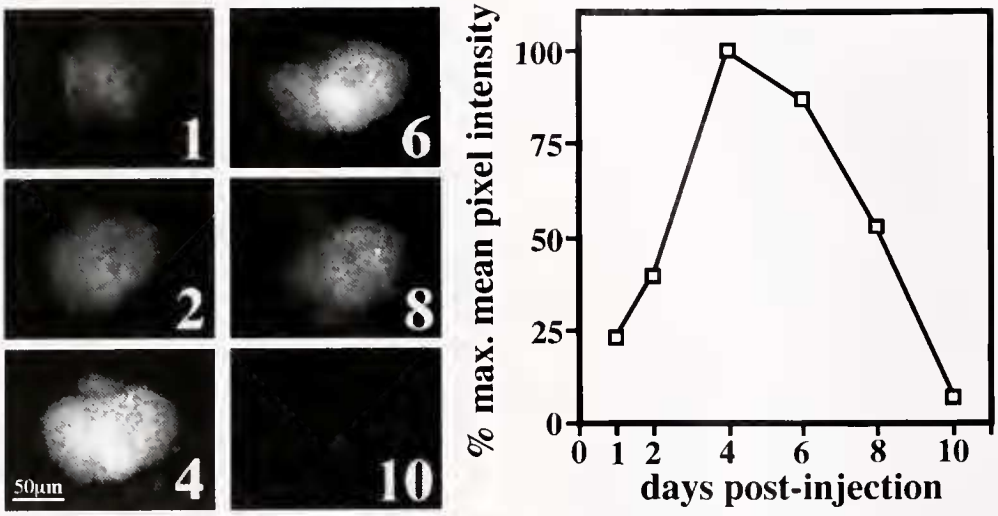

Figure 4. Time course of GFP expression after injection of cRNA. (Left) A single neuron injected with GFP-encoding cRNA was photographed, using a confocal fluorescent microscope, at various time points (in days) following injection. (Right) The intensity of fluorescence increased to a maximum at day 4, then declined to background at day 10.

by day 10 (Fig. 4). Other cells injected with this cRNA showed similar patterns of expression.

As the level of GFP fluorescence declined from its peak, its distribution in injected cells became patchy. In a few injections, GFP distribution was uniform in the cytoplasm but later became punctate, and the protein product appeared to be excluded from certain areas of the cytoplasm. In many cases, GFP fluorescence was seen in the primary neurite leaving the cell body (Fig. 5). However, we were unable to detect a GFP signal in deeper layers of the neuropil, in connectives, or in peripheral nerves.

Induction of β-galactosidase expression. Injection of the cRNA for β-galactosidase into cell somata resulted in enzyme activity detectable in cultured neurons within 2 days. Expression of this enzyme was determined by using the fluorogenic substrate fluorescein di-(β-D-galactopyranoside) to measure activity. Hence, we did not directly measure the amount of protein synthesized. Since enzyme activity probably was directly related to the amount of protein present, levels of expression of β-galactosidase apparently peaked between 6 and 9 days after injection. Strong β-galactosidase activity was still easily detectable in cultured ganglia 10 days after cRNA injection (Fig. 6). The fluorescence intensity increased rapidly and close to linearly in a β-galactosidase-expressing cell following addition of the fluorogenic substrate. Since the fluorescent cleavage product leaves the cells within a few hours, the same cells can be repeatedly tested for β-galactosidase activity on consecutive days.

Injection of DNA into lobster neurons

We attempted to induce reporter gene expression by the cytoplasmic microinjection of plasmid DNA. Identified cells were injected with DNA constructs containing three different promoters: the human CMV immediate early enhancer/promoter; *Drosophila melanogaster hsp70;* and *D. melanogaster elav* promoter driving either GFP or β-galactosidase. Injections of low concentrations of DNA (below 1 μg/μl in the electrode) had no detectable toxic effect on cells, whereas injections of DNA at concentrations above 1 μg/μl led to cell death. Most cells injected with higher levels of DNA became opaque and autofluorescent within 1 to 2 days (see Fig. 3), and all such cells showed low or no resting membrane potentials, indicating that they were dead or in the process of dying. In no case (low or high concentrations of DNA), however, did we observe any protein product expression.

Discussion

We have used an organ culture method to maintain the isolated central nerve cord of the lobster for up to 7 weeks, a significant extension beyond the 1 to 2 days previously possible. During this time RNA and protein synthesis, as well as physiological activity in identified neurons, appeared normal. This technique makes possible a range of long-term experiments, including study of the effects of pharmacological and hormonal treatment on the central nervous system, and the effects of manipulation of gene expression in central neurons.

Long-term culture of lobster ventral nerve cord

Ventral nerve cord preparations of crustaceans are valuable for exploring central circuitries because they allow absolute identification of neurons (see Otsuka *et al.*, 1967; Kennedy *et*

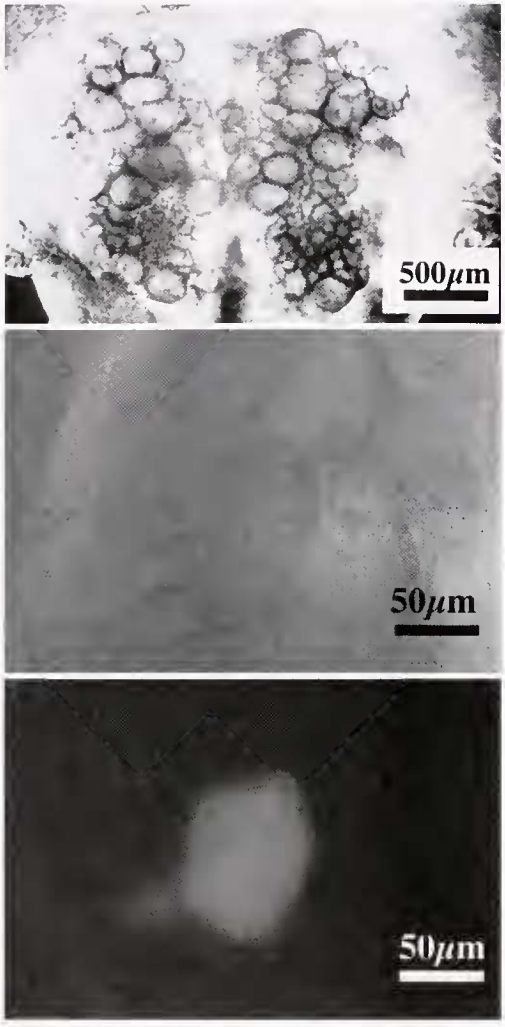

Figure 5. Lobster neuron injected with GFP cRNA. (Top) A desheathed lobster central ganglion at low magnification. (Middle) High-magnification view of an injected cell (center) under bright field illumination. (Bottom) The same view in dark field, showing fluorescent signal from the expressed GFP protein. The signal fills the injected soma and can be seen in the proximal section of the primary neurite as it descends into the neuropil.

al., 1969; Roberts *et al.*, 1982; Beltz and Kravitz, 1987; Ma *et al.*, 1992; Yeh *et al.*, 1996; Hörner *et al.*, 1997). Once exposed by removal of the connective tissue sheath, the somata of these neurons are easily visible and accessible to microelectrodes. Cells occur in predictable arrangements and can be unambiguously identified by their position, size, and activity, and by backfiring their axons from roots or connectives (Otsuka *et al.*, 1967). In our laboratory, preparations of this type have been used to explore the roles of amines in the neural networks involved in postural regulation (Harris-Warrick and Kravitz, 1984; Beltz and Kravitz, 1987; Ma *et al.*, 1992; Weiger and Ma, 1993). In crayfish, similar preparations have been used to define changes in synaptic properties accompanying changes in social status at particular synaptic sites (Yeh *et al.*, 1996).

In the organ culture system described here, we showed that neurons are suitable for electrophysiological experimentation for at least 7 weeks. We used only one example for illustration. In Figure 1 we showed that the large inhibitory motoneuron I2 could be activated by backfiring its axon at a distance of several centimeters from the cell body, and that interneurons upstream of I2 still could relay signals *via* the release of transmitters. Moreover, in single I2 cells dissected from long-term organ cultures of ganglia, the intracellular levels of GABA were comparable to those found in cells dissected from fresh preparations.

The autoradiographic studies demonstrated that neurons in ganglia cultured for 49 days still synthesized RNA (Fig. 2) and protein (data not shown). In addition, at a light microscopic level, the cytology of cultured neurons appeared normal, except for a somewhat more frequent vacuolar appearance of cell somata, and for larger spaces between cells and neuropil processes. These differences may result from looser cell packing in the excised and desheathed ganglia, from degeneration of sensory fibers from the periphery (Barker *et al.*, 1972), or both.

Cells that do not survive in organ culture appear dark with light microscopy and their autofluorescence is weak, making them easy to distinguish from surviving cells. Autofluorescence of unhealthy or dead cells has been reported elsewhere (Linnik *et al.*, 1993; Kosslak *et al.*, 1997), and is a useful way to prevent making comparisons between these cells and healthy cells in the cultures (O'Brien *et al.*, 1995). It is important to identify such cells so that their fluorescence is not confused with that of experimental markers like GFP.

GFP and LacZ *are suitable expression markers in lobster neurons*

GFP and *LacZ* (the gene encoding β-galactosidase) have been used as reporter and marker genes in studies involving a wide variety of organisms (see Chalfie, 1995). Lobsters and crayfish (see Dearborn *et al.*, 1998) now can be added to the list of organisms capable of expressing these proteins. The studies presented here, and those reported by Dearborn *et al.* (1998), suggest that it may be possible to use GFP and β-galactosidase as injection markers, as reporters for transcriptional studies, and as tags that can be fused to proteins under study in crustacean systems. To illustrate the latter, in preliminary studies (G. Ganter, unpubl. obs.), we have found that intracellular injections of cRNAs encoding a human β-2 adrenergic receptor/GFP fusion and a lobster amine receptor/GFP fusion result in fluorescent signals. Although different cRNAs might show differences in their rates of translation, thus far all cRNAs that we have injected have been expressed. Indeed, the different times we noted for peak detection of β-galactosidase and GFP could be due to differences in the translation rates of these two transcripts.

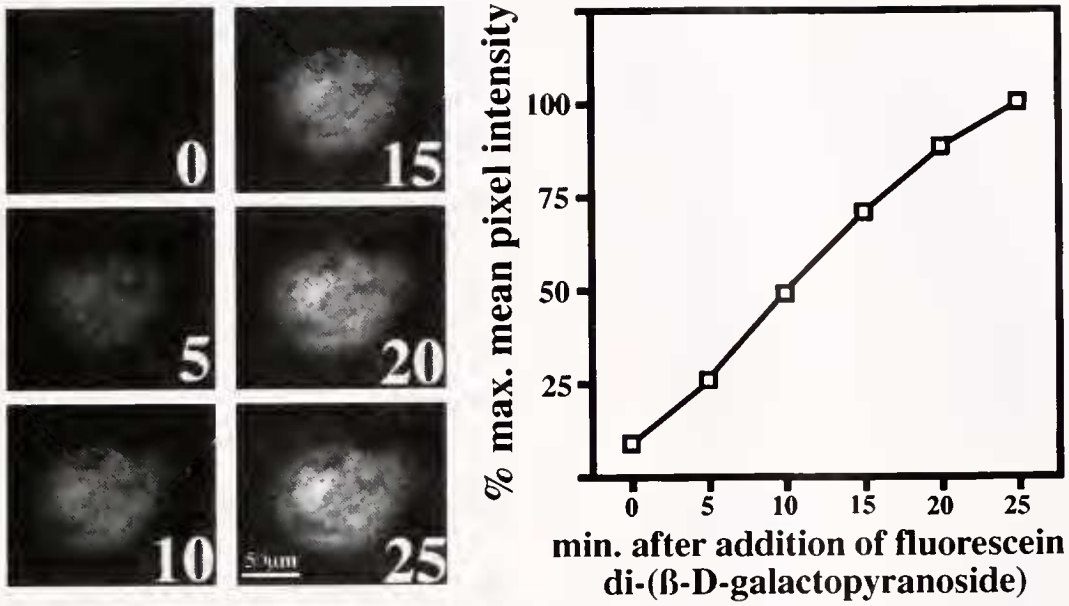

Figure 6. β-galactosidase activity in a lobster neuron maintained 10 days in organ culture after injection with *LacZ* cRNA. β-galactosidase activity was detected in a living injected cell by addition of a fluorogenic substrate, fluorescein di-(β-D-galactopyranoside). (Left) The cell was photographed, using a confocal fluorescent microscope, at various time points (in minutes) following addition of the substrate. (Right) The intensity of fluorescence increased almost linearly.

Future uses of the organ culture method

Long-term organ culture offers a controlled environment in which to perform experiments that were difficult to carry out in short-term studies using isolated nerve cords. This should allow us to explore the consequences of applying test substances to central ganglia in more biologically relevant time frames. For example, the lobster molting hormone, 20-hydroxyecdysone, is likely to have actions at both membrane and genomic levels (see Zakon, 1998). The genomic effects may take days to bring about observable changes at synaptic or circuit levels. The organ culture system offers enough time for such changes to be seen, in an environment in which tissues will continue to synthesize the RNA and protein required to trigger the changes. Long-term drug effects relating to aminergic function also can be examined in the cultured ganglia. At present we are particularly interested in chronic exposure of ganglia to Prozac (fluoxetine). In behavioral studies, acute exposure to Prozac has little effect on agonistic behavior in lobsters (Huber *et al.*, 1997); in contrast, chronic exposure increases the amount of time that animals are willing to fight (A. Delago, unpubl. obs.).

Another exciting application of the organ culture method is in the experimental manipulation of gene expression in identified neurons. The extended time frame makes it possible to examine the effects of gene expression on the physiology of cRNA-injected neurons and to analyze cloned genes in their native environment. For example, the serotonin-containing neurosecretory neurons of lobster appear to lack the mRNA for the *shab* form of the potassium channel (H. Schneider, unpubl. obs.). It would be interesting to express *shab* in these neurons, and then examine the consequences of this manipulation on the intrinsic properties of the injected cell and on the network in which it functions. Other applications could include injections of sense, antisense, or double-stranded RNAs coding for particular proteins into cells to ask how such manipulations alter function. These and other applications await further exploitation of this important system.

Summary and conclusions

We describe an organ culture method that maintains isolated lobster ganglia in viable states for up to 7 weeks. We have validated the method, showing that evidence of gene expression and electrophysiological activity persist throughout the culture period. Applications include long-term experiments to examine the consequences of chronic treatment with pharmacological or hormonal reagents and of changes to the levels of expression of particular genes in single neurons and in networks of neurons. The ability to introduce genes into lobster nerve cells should allow analysis of the function of these genes in their native environment, narrowing the gap between molecular methods and the study of physiology in this important system.

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